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The Efficacy of a Topically Applied Imidacloprid 10%/Moxidectin 2.5% Formulation (Advocate®, Advantage® Multi, Bayer) against Immature and Adult *Spirocerca lupi* Worms in Experimentally Infected Dogs

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Abstract

This study investigated the efficacy and safety of an imidacloprid 10%/moxidectin 2.5% spot-on combination (Advocate®, Advantage® Multi, Bayer) against immature and mature stages of *Spirocerca lupi* in experimentally infected dogs. 24 dogs were allocated to 3 groups and infected with approximately 10 L_3 larvae of *S. lupi* orally on study day (SD) +2, +14, +28 and +42. Group 1 remained as untreated control group. Group 2 dogs were treated on SD –28, 0, and thereafter monthly until Day 280 (12 treatments). Group 3 dogs were treated weekly on 19 occasions starting on SD +170. The dosage for all treatments was the licensed dose of 10–25 mg imidacloprid/2.5–6.25 mg moxidectin per kg body weight. All dogs were examined on SD +169 or +176 by endoscopy. Group 3 dogs were additionally examined approximately every two weeks up to Day 296. On Day +308 or +310, all dogs were necropsied to recover *S. lupi* worms and to quantify lesions in the thoracic aorta and oesophagus. Dogs in the control group were adequately infected with *S. lupi*, demonstrated by the extensive damage to the thoracic aorta, the nodules in the oesophagus and the large numbers of worms recovered. In total 144 worms were collected (geometric mean of 16.8 worms per dog). Dogs in group 2 had no or very slight damage to the thoracic aorta and no nodules or worms in the oesophagus, indicating 100% efficacy of the monthly treatments. Dogs in group 3 were also adequately infected, showing nodules in the oesophagus before initiation of weekly treatment, and at necropsy extensive damage was seen

in the thoracic aorta. After treatment, three dogs of 8 still had a few nodules and in total three worms (GM of 0.25 per dog) were recovered, demonstrating an efficacy of 98.5% against adult *S. lupi*. All dogs tolerated the treatment well and no treatment-related adverse events occurred.

Introduction

Spirocerca lupi is a spirurid nematode parasite, the adults of which are usually found in fibrous nodules in the wall of the oesophagus of dogs and other canids, although cats and other wild felids may sometimes also act as final hosts (Bailey 1972; Taylor et al. 2007). In general, large breed dogs appear to be most at risk of infection, especially hunting dogs, and Labrador retrievers appear to be more susceptible than other breeds (Mazaki-Tovi et al. 2002; Mylonakis et al. 2001), but this may be linked to specific lifestyles providing greater or lesser exposure to the parasite (van der Merwe et al. 2008). There is no age or sex predilection, although animals under six months of age, even if infected, will not show clinical signs of infection because oesophageal spirocercosis develops gradually as the worms mature over an approximately six months period (van der Merwe et al. 2008).
Although *S. lupi* appears to have a worldwide distribution (Lavy et al. 2003), it occurs most often in warmer tropical and subtropical climates (Bailey 1972; Berry 2000; van der Merwe et al. 2008). The widespread importance of this parasite is demonstrated by numerous prevalence studies conducted in a number of countries over the last 5 decades (Brodey et al. 1977; du Toit et al. 2008; Haralabidis et al. 1988; Lobetti 2000; Murray 1968; Mylonakis et al. 2001; Oliveira-Sequeira et al. 2002; Pandey et al. 1987; Pence and Stone 1978; Ramírez-Barrios et al. 2004).

Fig. 1a SEM of the anterior end of an adult *Spirocerca lupi* showing the hexagonal mouth, cephalic papillae (arrows) and cervical papilla (arrow head)

Fig. 1b *Spirocerca lupi* adult specimen collected from one dog post mortem following rupture of the aorta (dog not part of the study population)

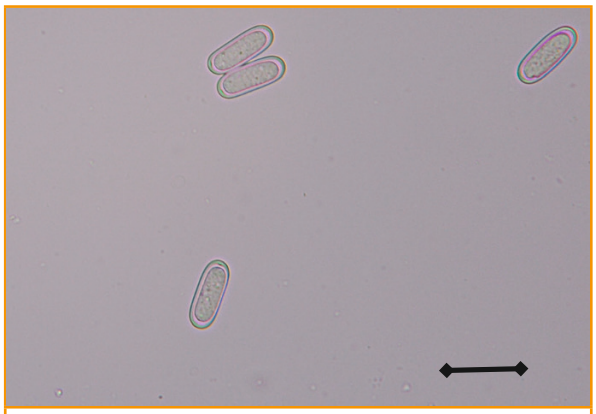

Fig. 1c *Spirocerca lupi* eggs as found during flotation procedure. Bar: 40 µm

The distribution of the parasite is dependent on the occurrence of the intermediate hosts, which include coprophagous beetles of the family Scarabaeidae (Bailey et al. 1963). Paratenic or transport hosts can also serve as sources of infection for the final host if ingested and infection may be transferred from one paratenic host to another via predation (Fox et al. 1988). Lizards, chickens, mice and a number of insectivorous vertebrates may act as paratenic hosts for the infective larvae (Bailey 1972; Bowman 2009; Taylor et al. 2007).

The ability of this parasite to utilise vertebrate paratenic hosts increases the availability of infective stages due to an increased number of potential intermediate hosts, as well as the relatively longer lifespans of such paratenic hosts. Similar roles of additional paratenic hosts are also reported for other parasites such as *Angiostrongylus vasorum*, where frogs prey on the intermediate host snail and widen the scope of prey for the final canid host. (Bolt et al. 1993)

After the intermediate respectively paratenic host is ingested, the encysted third-stage larvae (L_3) excyst in the stomach before migrating through the mucosa. The larvae then migrate within the walls of the gastric and gastroepiploic arteries to the aorta and then cranially into the thoracic aorta; this portion of the migration usually taking approximately 10 days (Bailey 1972; van der Merwe et al. 2008). Here the larvae will remain for up to three months, undergoing two further moults to emerge as young adults, subsequently migrating directly into the caudal oesophageal wall, penetrating the mucosa before returning to and settling in the submucosa or adventitia to complete their maturation and life cycle (Bailey 1972; van der Merwe et al. 2008).

Adult worms (Figs. 1a and 1b) are spiralled and pink to blood red in colour, the males growing up to 55 mm and the females up to 80 mm in length (Soulsby 1982; Taylor et al. 2007). The females pass eggs (Fig. 1c) through the perforation in the mucosa, where they can be passed out of the body by vomiting or by swallowing and subsequent defaecation; subsequent ingestion of the eggs by coprophagous beetles starts the life cycle again (Fig. 2).

The pathological changes associated with *S. lupi* infections are brought about by the migration of the larvae through the various tissues and organs as well as their persistent presence in the body (Berry 2000; Gal et al. 2005). The most significant lesions include aortic aneurysms, posterior thoracic spondylitis and oesophageal nodule formation which often progresses to metastatic sarcoma (Bailey 1963; Dvir et al. 2010).

The migration of the larvae within the walls of blood vessels results in haemorrhage, necrosis and the exudation of neutrophils locally (van der Merwe et al. 2008). The above-mentioned lesions usually

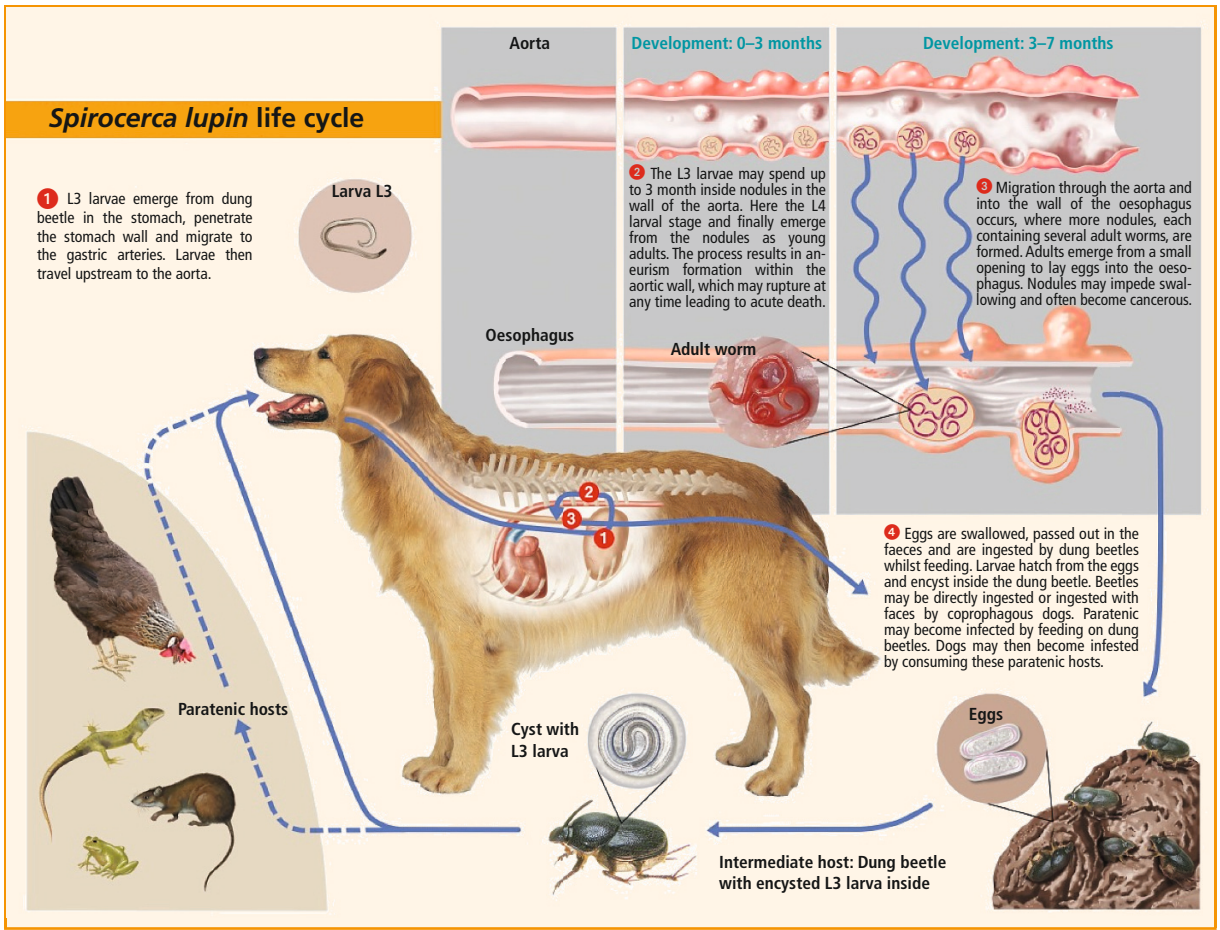

Fig. 2 Schematic representation of the life cycle of *Spirocerca lupi*

heal completely, except in the thoracic aorta where migrating larvae damage the elastic and muscle layers, causing subsequent degeneration, fibrosis, calcification and ossification (Bailey 1963; Bailey 1972; van der Merwe et al. 2008). These processes culminate in scarring of the intima and varying degrees of aortic aneurysm formation (Johnson 1992). The aortic aneurysms, considered pathognomonic for *S. lupi* infections, are frequently the cause for acute death due to aortic rupture and fatal haemorrhage (Bailey 1963, 1972).

Subclinical infections aside, antemortal clinical signs in dogs infected with *Spirocerca* are varied and may include coughing, regurgitation, anorexia, weight loss, vomiting, dysphagia, salivation, inhalation pneumonia, paraparesis, back pain, melaena, anaemia and central nervous system involvement (Dvir et al. 2001; Du Plessis et al. 2007; van der Merwe et al. 2008). A diagnosis of spirocercosis in the dog may be aided by the presence of various combinations of the above-mentioned symptoms as well as the presence of *S. lupi* eggs in the stool, demonstrated by a modified faecal flotation technique (Markovics and Medinski 1996), reported to increase the diagnostic sensitivity of the test. Various radiographic changes such as a caudal oesophageal mass with accompanying thoracic vertebral periosteal reactions are also supportive of a diagnosis (Dvir et al. 2001), but direct visualisation of oesophageal nodules or neoplastic masses by means of an endoscopic examination remains the most sensitive method of diagnosis (van der Merwe et al. 2008).

Up until the time of this study, the most effective treatment for non-neoplastic spirocercosis appeared to be doramectin, administered subcutaneously in varying regimens (Berry 2000; Lavy et al. 2002; van der Merwe et al. 2008), although concerns exist about its extra-label use, mode of administration and breed-specific toxicity (Kok et al. 2010). Orally administered milbemycin oxime has also been shown to have some efficacy against *Spirocerca lupi* (Kelly et al. 2008; Kok et al. 2010, 2011).

The prevalence of *Spirocerca lupi* in many parts of the world is ever increasing (Kok et al. 2010) and the need exists for a licensed, cost-effective product which will provide adequate preventative and therapeutic efficacy, demonstrating effectiveness against the migrating immature stages as well as the encapsulated adult stages of the parasite. A study conducted on Réunion Island has indicated that a topically applied formulation consisting of imidacloprid 10%/moxidectin 2.5% can be safely and successfully used to prevent canine spirocercosis in puppies (Le Sueur et al. 2010), but the preventative and therapeutic ability of this formulation needed to be further investigated. A study was therefore designed to investigate the preventive efficacy (efficacy against immature stages), the therapeutic efficacy (efficacy against mature stages) and the safety of an imidacloprid 10%/moxidectin 2.5% spot-on formulation (Advocate®, Advantage® Multi, Bayer) against *Spirocerca lupi* in experimentally infected dogs.

Materials and methods

The study took the form of a monocentric, randomised, parallel-arm, partially blinded, negative controlled, terminal efficacy study and was conducted on the premises of Clinvet International, a contract research organisation located in Bloemfontein, South Africa. The study was conducted in compliance with the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline (GL) 9 on Good Clinical Practice.

A total of 24 purpose-bred, mixed breed dogs between the ages of 7 and 14 months were used. All animals weighed between 11 kg and 22 kg and were determined to be healthy and free of parasites by veterinary personnel at the start of the study. All were treated with a suitable anthelmintic, not from the macrocyclic lactone family, at least 14 days before the onset of group allocation on Day –29. Each animal was identified by means of an implanted electronic transponder.

The South African animal welfare regulations, detailed in the South African National Standard "SANS 10386:2008 *The care and use of animals for scientific purposes*", were strictly adhered to and the study protocols were submitted to the Clinvet Animal Ethics Committee for approval. The composition of this committee was in compliance with the above-mentioned national standard.

Study design

The study was conducted in three phases in order to keep the infection episodes adequately spaced, so that the number of infective larvae required at each infection episode would not be excessive. The study population (n = 24) was divided into 3 study groups using randomisation based on gender and body weight. After being assigned to a group, the animals were then randomly assigned to phase 1, 2 or 3 of the study. In phases 1 and 2, each group consisted of 3 animals (9 animals in total). In the third phase, each group consisted of 2 animals (6 animals in total). The layout of the study was the same for all phases and all animals were individually housed in order to reduce the risk of cross-contamination with test product as well as facilitate daily health observations and collection of stools for faecal egg counts. All animals were regularly allowed individual access to runs in order to exercise and were treated monthly with an effective acaricide (Advantix®, Bayer) for ectoparasite control. Each animal was dosed with a suitable oral anthelmintic (not containing a macrocyclic lactone) and vaccinated prior to the start of the trial.

The Investigational Veterinary Product (IVP) in this study was Advocate® (Bayer), a topically applied endectocide containing imidacloprid 10 %/ moxidectin 2.5 %. The treatment regimen of each group is demonstrated in Table 1.

Group 1 served as the negative control group that remained untreated. Group 2 received monthly treatments with the IVP throughout the study, beginning 28 days prior to the first artificial infection with *S. lupi*, and was used to determine preventative efficacy. Group 3 received weekly treatments with the IVP from 170 days post infection (p.i.), subsequent to confirmation of the presence of *S. lupi* nodules in the oesophagus, and treatment continued until 2 weeks prior to termination of the study. This group was used to determine therapeutic efficacy. Each animal received a minimum of 10 mg/kg (milligrams per kilogram body weight) imidacloprid and 2.5 mg/kg moxidectin with each dose of Advocate® that was applied. Table 1 shows the dosages applied in accordance with the manufacturer's instructions.

All animals were examined endoscopically on Day +169 or +176 to determine whether or not oesophageal nodules (i.e. oesophageal spirocercosis) had already developed; thereafter animals in group 3 were endoscopically examined approximately every 2 weeks and the presence, size and position of any nodules present were recorded.

Faecal samples were collected once weekly from Day +170 and examined for the presence of *S. lupi* eggs. Table 2 gives a timeline depicting important events pertaining to the study.

As treatment regimen and examination procedures (e.g. endoscopy schedules) differed between the respective groups, no blinding procedures were applied during most of the study. A blinding procedure was, however, applied during the necropsy procedures. Blinding codes were allocated to individual animals to ensure that persons performing the actual necropsy examinations and assessments were unaware of group allocations.

Induced infection of dogs

All animals in the study were challenged with approximately 10 infective L_3 larvae on Days +2, +14, +28 and +42 (+/–2 days) of the study. The infective larvae were dissected out of naturally infected scarabaeid beetles, collected from an area in South Africa known to be endemic for spirocercosis. The excysted larvae were kept in saline and were dosed to the dogs within 24 hours of their recovery.

After being fasted for approximately 12 hours, each dog was dosed with the 10 infective larvae, suspended in 2 ml (millilitres) of saline, by means of a stomach tube. After the saline containing the larvae was dosed, the stomach tube was rinsed through with a further 10 ml of saline before

Table 1 Different group treatment regimens

Group	Active ingredients	Dosages	Dose	Days treated	Number of dogs
1		Untreated control			8
2	Imidacloprid 10 %/moxidectin 2.5 % spot on	2.5 ml/dog weighing > 10 up to 25 kg 4.0 ml/dog weighing > 25 up to 40 kg	10–25 mg imidacloprid / 2.5–6.25 mg moxidectin per kg b.w.	Study day –28, 0, +28 and then monthly up till within five weeks prior to termination of the animal phase	8
3	Imidacloprid 10 %/moxidectin 2.5 % spot on			Weekly from approximately 6 months post infection (i.e. Day +170) up to approximately 2 weeks before termination of the animal phase	8

Table 2 Study schedule

Day	Activity
–32	Animal phase starting date
–29	Allocation to groups
–28	1st IVP treatment, group 2
+2	1st infection with 10 *S. lupi* L_3
+42	Last infection with 10 *S. lupi* L_3
+169 +176	First endoscopy, all groups
+170	1st Faecal egg counts 1st IVP treatment, group 3
+ 280	Last IVP treatment, group 2
+296	Last IVP treatment, group 3
+306–308	Last faecal egg counts
+308/+310	Necropsy; examination of aorta and oesophagus; worm recovery

removal. Each animal was treated with a low dose of acetylpromazine maleate approximately 20 min before the procedure to provide for a mild degree of muscle relaxation combined with an anti-emetic effect. The procedure was performed by a veterinarian and lasted no longer than 30 seconds in each case. During and for at least 1 hour directly after infection, the dogs were observed continuously for any vomition, which was recorded if present.

In spite of premedication, with acetylpromazine maleate all dogs infected with *S. lupi* larvae vomited after introduction of the larvae, all within the first hour with one exception. In most instances, the vomit was licked up again by the dog. It was known from previous experience that few, if any, larvae were ever present in the vomit and that the vomiting would have little effect on the potential success of establishment of the infections.

Assessment

Endoscopic examination

Dogs in all groups were examined endoscopically under general anaesthetic on Day +169 or +176. From Day +183 onwards, only the dogs in group 3 were endoscopically examined, initially every 2 weeks but later amended to allow slightly longer intervals between examinations; this decision was taken because some of the animals started to experience delayed recovery times and increased extensor rigidity after general anaesthetic, and the concern existed that this was due to the high frequency of exposure to general anaesthetic.

Each animal was pre-medicated 10 minutes prior to the procedure with intravenous diazepam and atropine and induced with intravenous propofol, titrated to effect. After intubation and inflation of the endotracheal tube cuff, animals were maintained on halothane inhalation anaesthesia in left lateral recumbence, with the head and neck extended, whilst the endoscopic examination took place.

The oesophageal mucosa was examined over its entire length up to the gastro-oesophageal orifice and the number, size and position of any nodules or nipple-like protuberances (van der Merwe et al. 2008) were recorded. Each video endoscopy was recorded digitally and a number of still images of various lesions were recorded.

Faecal examination

Since animals were individually housed, individual faecal sampling was possible and *S. lupi* egg counts were conducted on all animals on a weekly basis from Day +170 onwards. A sugar flotation method, as described by Marcovics and Medinski (1996), was used and counts were expressed as eggs per gram of faeces (epg).

Necropsy

Necropsy of the animals in phase 2 took place on Day +308 and on Day +310 for phases 1 and 3. The animals had food withheld for 16 to 20 hours before humane euthanasia using an overdose of pentobarbitone, administered intravenously. At

Table 3 Aortic damage score criteria

Score	Description
1	No lesions visible on inside
2	Inflammatory/puckered lesions only just visible
3	Two to three lesions clearly visible, no aneurisms
4	Several distinct inflammatory lesions; parasite bore tracks are often visible; aneurisms just start developing
5	Distinctly damaged area of aorta; all of the above evident
6	Worst case scenario: aorta severely damaged over a large area; several aneurisms evident

necropsy, an extensive post-mortem examination of the thoracic aorta and oesophagus was conducted and all lesions described and quantified as far as possible. The size of *S. lupi* lesions in the oesophagus and their position relative to the diaphragm was measured. Photographic documentation was also compiled.

All nodules were opened and the contents washed through a 0.15-mm sieve. After backwashing, the contents of the sieve were macroscopically examined and all helminths and helminth fragments removed and preserved. A blinding procedure, which included coding of tissue and organ samples removed, was followed to ensure that the persons performing the parasite and lesion assessments were unaware of group allocations. The number of nodules on both oesophagus and thoracic aorta were recorded and the diameter of such nodules was measured as far as possible. Furthermore, the severity of damage to the aorta was scored using the criteria laid out in Table 3.

Calculation of efficacy

The main numerical assessment for statistical analysis was the number of worms recovered at necropsy in the treated groups (groups 2 and 3) compared to the control group (group 1). Percentage efficacy, as a measure of a treatment effect to prevent encapsulation, was the primary endpoint for group 2. Percentage efficacy, as a measure of a treatment effect against encapsulated adults, was the primary endpoint for group 3. Adequacy of infection and a significant difference in worm counts between the treated and control groups were required to claim a treatment effect. Evaluation of the adequacy of infection of the dogs was based on parasitological criteria.

The treated groups were also statistically compared to the control group with regard to the reduction in faecal egg output. The percentage reduction, as a measure of treatment effect, was a secondary endpoint.

Data on the size and numbers of lesions were used mainly descriptively, as were data resulting from endoscopic examinations.

The efficacy of treatment with IVP (i.e. either to prevent encapsulation of *S. lupi* or efficacy against encapsulated adults) was reflected in the number of *S. lupi* worms recovered at necropsy in the treated groups compared to the control group.

The formula was:

$$\% \text{ Efficacy} = (N_2 - N_1)/N_2 \times 100$$

N_1 = Arithmetic Mean (AM) or Geometric Mean (GM) of the number of *S. lupi* recovered at necropsy for group 2/group 3

N_2 = AM or GM of the number of *S. lupi* recovered at necropsy for group 1 (control)

For calculations of efficacy, arithmetic and geometric means were used. The efficacy based on

geometric means was considered primary. To allow for data sets with zero values, all data were transformed to (n + 1), geometric means calculated and 1 subtracted from the result to obtain a reasonable estimate of the geometric mean.

To better fulfil the requirements for normality and homogeneity of variance, each individual value used in statistical comparisons (e.g. worm numbers) was transformed to log (n + 1) for further analysis.

For comparison between groups, an analysis of variance (ANOVA) with a treatment effect was applied to the numbers of worms after a logarithmic transformation of the (worm + 1) counts. Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using SAS Version 8.02 or higher.

The efficacy of treatment with the IVP to reduce faecal egg output in dogs in the treated groups compared to those in the control group was expressed as percentage reduction, calculated for each week following Day +170.

The formula was:

$$\% \text{ Reduction} = (E_2 - E_1)/E_2 \times 100$$

E_1 = Arithmetic (AM) or Geometric Mean (GM) of the particular week epg value for group 2 or group 3

E_2 = AM or GM of the particular week epg value for group 1 (control)

The percentage reduction was analysed statistically in the same way as the efficacy calculations described above.

Results

Endoscopic examination

The untreated control dogs in group 1 were examined only on Day +169 or +176 and not again. All dogs in this group had one to six nodules in the oesophagus, confirming the success of establishment of *S. lupi* following experimental infections.

The dogs in group 2, which received monthly treatment with the IVP starting on Day –28, had no nodules in the oesophagus on Day +169 or +176. These dogs were also not examined endoscopically again.

The dogs in group 3 were also examined on Day +169 or +176 and regularly thereafter, initially at 14-day intervals but later with longer intervals as discussed earlier. Seven of the eight dogs in group 3, treated for the first time on Day +170, had one to six nodules in the oesophagus on Day +169/+176, thus also confirming the success of establishment of the *S. lupi* infections. One dog had no visible nodules in the oesophagus and it remained negative throughout the examination period. In three other dogs, the number of nodules remained more or less the same (2 to 3) throughout the examination period. In the four remaining dogs, the number of nodules decreased from 4–6 initially to 1 or 2 at the end of the examination period. On the last day of examination (Day +281 or +295), 1–3 nodules were still observed endoscopically in seven of the eight dogs.

Faecal egg counts

Figure 3 shows the pattern of change in weekly geometric mean (GM) *S. lupi* epg values for groups 1 and 3 from Day +170 up to the end of the study.

In the untreated group 1, egg numbers were initially low and found sporadically, not always in the same dogs. From about Day +208 egg numbers were larger and eggs were found consistently in six of the eight dogs. From about Day +268 egg numbers increased and eggs were mostly found in all dogs on all occasions. Epg values were mostly below 50, but in one particular animal, the count value often exceeded 100 epg after Day +261 and on one occasion even reached 326 epg.

In group 2, treated monthly from Day –28, no eggs were ever found throughout the study.

In group 3, treated weekly from Day +170, epg values were initially similar to those found in group 1, but, as the study progressed, the values became lower and egg findings more sporadic. On Days +170/+177, eggs were found in the faeces of six of the eight dogs. From Day +208 eggs were found

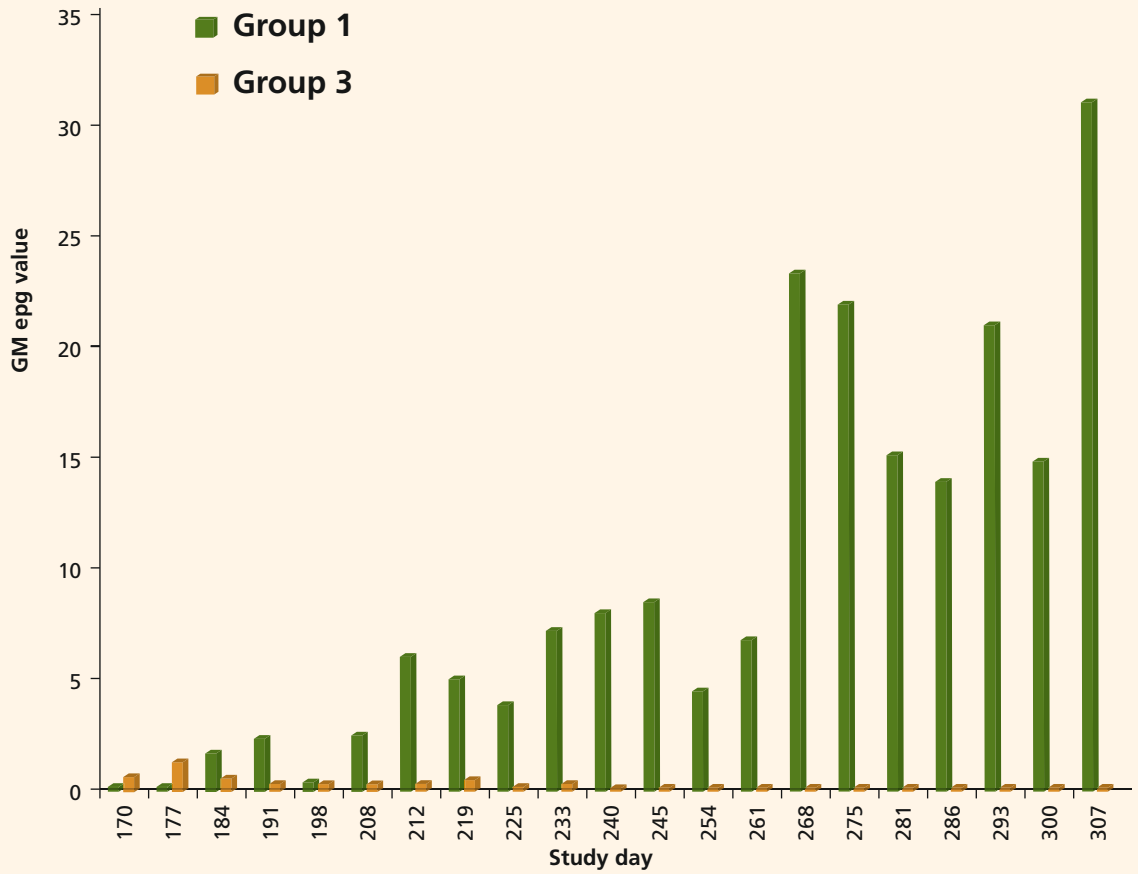

Fig. 3 Faecal *Spirocerca lupi* egg counts were conducted from approximately 6 months (study day +170) after experimental infection of the dogs. Egg counts are expressed as eggs per gram of faeces (epg) and the graph shows mean epg values, determined approximately weekly, for dogs in groups 1 and 3. No *S. lupi* eggs were present in the faeces of dogs in group 1

in the faeces of only two dogs, from Day +233 in the faeces of only one dog and from Day +254 eggs were no longer found in the faeces of any of the dogs (Fig. 3). The percentage reduction in egg output when compared to the control group, although variable, was initially over 85% and progressed to over 90% from Day +208 and 100% from Day +254 onwards.

Lesions in the aorta recorded at necropsy

In the untreated control group 1, the number of lesions on the inside of the thoracic aorta varied in number from 5 to 17 between individual dogs (median 11.5; geometric mean 10.7). Typical nodules were mostly absent and the lesions remaining were made up of puckered areas and small to large aneurisms. No *S. lupi* worms remained in the wall of the aorta. The scores awarded as a means of quantifying the damage to the aorta are depicted in Table 4 and varied from 3.5 to 5 (median 4.25; geometric mean 4.23).

In group 2, treated from Day –28, no sign of any lesions could be found in the thoracic aorta of seven of the eight dogs, which were awarded damage scores of 1. In one dog, two small puckered areas were seen around an arterial branch, so small that microscopic examination was necessary, which still failed to make clear whether or not the lesions could

Table 4 Comparative aortic damage at necropsy

Group	Treatment	No of lesons (GM)	Damage score (GM)
1	Untreated control group	10.7	4.23
2	Monthly prevention from Day –28	0.1[a]	1.06[c]
3	Weekly therapy from Day 170	10.4[b]	4.43[d]

[a] p value for difference to group 1 and 3: 0.0003
[b] p value for difference to group 1: 0.2077
[c] p value for difference to group 1 and 3: 0.0003
[d] p value for difference to group 1: 0.4160

Table 5 Comparative lesions in the oesophagus at necropsy

Group	Treatment	No of nodules (GM)	Size of nodules
1	Untreated control group	2.1	Mostly large
2	Monthly prevention from Day –28	0[a]	0[c]
3	Weekly therapy from Day 170	0.4[b]	Average[d]

[a] p value for difference to group 1: 0.0002; p value for difference to group 3: 0.038
[b] p value for difference to group 1: 0.002
[c] p value for difference to group 1: < 0.0001; p value for difference to group 3: 0.036
[d] p value for difference to group 1: < 0.0001

have been caused by *S. lupi* larvae (median 0.0; geometric mean 0.1). The cause of the lesions remained inconclusive but a damage score of 1.5 was awarded (median 1.0; geometric mean 1.06).

In group 3, treated from Day +170, the damage to the aorta closely resembled that in group 1. The number of lesions varied from 6–16 (median 10.0; geometric mean 10.4), and damage scores varied from 3–6 (median 4.5; geometric mean 4.43).

It is evident that groups 1 and 3 did not differ significantly with regard to both parameters measured and the damage to the aorta was similar for the two groups. Damage to the aorta was significantly less in group 2 than in either of the other two groups (Table 4).

Figures 4a to 4c show some examples of the thoracic aorta of individual dogs where either no damage or various degrees of damage occurred, as well as the scores that were awarded.

Lesions in the oesophagus recorded at necropsy (Table 5)

In the untreated control group 1, nodules were present in the oesophagus of all dogs, varying in number from 1 to 3 (median 2.5; geometric mean 2.1). The nodules were mostly large and all of them contained adult *S. lupi* worms.

In group 2, treated from Day –28, no sign of any nodules could be found in the oesophagus of any of the dogs.

In group 3, treated from Day +170, nodules were found in the oesophagus of three dogs (median 0.0; geometric mean 0.4) In two of the dogs, with only one nodule each, the nodules were of average size and contained one or two worms. The other dog had two very small nodules in the oesophagus and these contained no worms.

The average nodule size was also compared between the three groups by the use of an ANOVA. It is evident that the oesophageal nodules found in group 3 were significantly smaller than those in the control group. Figures 5a and 5b show examples

Fig. 4a Aorta (from a dog in group 2 treated monthly from Day –28) with no lesions awarded a damage score 1

Fig. 4b Aorta (from a dog in group 3 treated weekly from Day +170) awarded a damage score 5. Many lesions, some of which are indicated by arrows, can be seen in the wall of the aorta

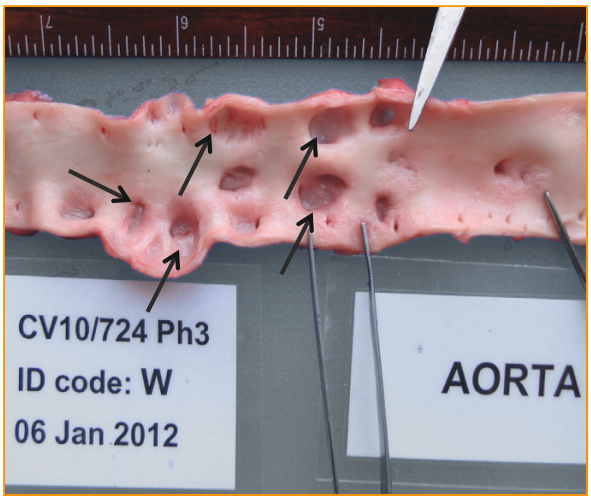

Fig. 4c Aorta (from a dog in group 3 treated weekly from Day +170) awarded a damage score 6. The wall of the aorta shows extensive damage with many large aneurisms, some of which are indicated by arrows

of oesophageal nodules seen from both the outside and the inside of the oesophagus from a dog in the untreated control group.

Worm numbers and calculation of efficacy

The total worm numbers recovered from oesophageal nodules of dogs in the three study groups at necropsy are shown in Table 6.

In the untreated control group 1, *S. lupi* worms were recovered from oesophageal nodules in all dogs. Total numbers of *S. lupi* recovered per dog varied from 10 to 31, geometric mean 16.9. In total 144 worms were recovered from the control dogs and this represented 45% of the 320 *S. lupi* L3 larvae with which the eight dogs were infected, closely corresponding to the 47% recorded by Bailey (1972). The dogs in the control group were thus adequately infected with *S. lupi* as a result of the experimental infections. Vomiting, which followed after dosing with the L3 larvae, evidently had little effect on the success of establishment of the parasites.

In group 2, treated from Day –28, no *S. lupi* worms occurred in the oesophagus.

In group 3, treated from Day +170, *S. lupi* worms were found only in the two dogs with the larger nodules, which harboured one and two worms, respectively (geometric mean 0.3).

Efficacy against encapsulation of immature *S. lupi* worms

Percentage efficacy, as a measure of a treatment effect to prevent encapsulation of the immature stages of *S. lupi*, was the primary endpoint for group 2. In this group, no *S. lupi* worms or any

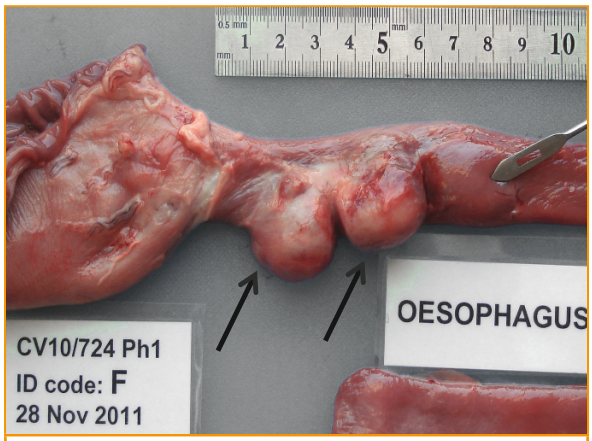

Fig. 5a Outer view of oesophageal nodules in a dog from group 1. Two large nodules, indicated by arrows, can be seen in the proximal part of the oesophagus; part of the stomach is visible on the far left. See also Fig. 5b

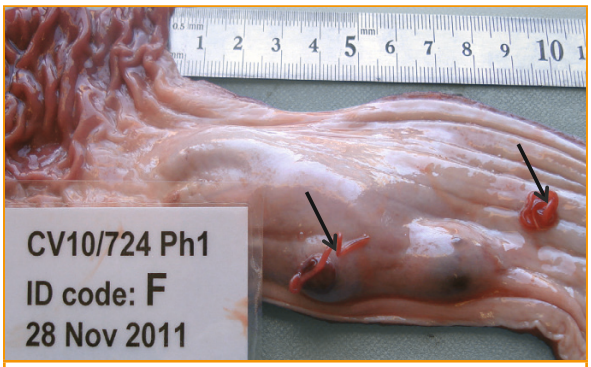

Fig. 5b The oesophagus shown in Fig. 5a opened so that nodules are viewed from the lumen side. Orifices connect the nodules to the oesophagus and these enable female worms to lay their eggs in the oesophageal lumen. One adult worm (arrow 1) can be seen protruding from an orifice and a second adult (arrow 2) lie free on the inner wall of the oesophagus

Table 6 Total number of *Spirocerca lupi* worms recovered from oesophageal nodules of dogs in groups 1–3 during necropsy

Group	Total *S. lupi* worms	GM	Percentage efficacy	
			Preventive	Therapeutic
1	144	16.39	Not applicable	
2	0	0.0	100.0	----
3	3	0.3	----	98.5

lesions caused by *S. lupi* during any stage of the life cycle were ever found in any of the dogs at necropsy. The treatment regimen was thus 100% effective in preventing encapsulation of *S. lupi* following experimental infection (Table 6).

Efficacy against encapsulated adult *S. lupi* worms

Percentage efficacy, as a measure of a treatment effect against encapsulated adults, was the primary endpoint for group 3. L_3 *S. lupi* larvae, introduced during the experimental infections, successfully established themselves in the thoracic aorta of the dogs in group 3, subsequently migrating to and forming nodules in the oesophagus of all but one of the dogs, as determined during endoscopic examinations. *S. lupi* eggs, albeit few, were shed in the faeces of six of the eight dogs in group 3 between Day +170 and +245. The above indicates that the infections were well established in the group 3 dogs by the time of the first IVP treatment on Day +170.

The geometric mean number of *S. lupi* recovered at necropsy from dogs in the untreated control group 1 was 16.9, compared to 0.3 in group 3. Efficacy of the treatment regimen was 98.5% (Table 6) against encapsulated adults in group 3 (97.9% based on arithmetic means).

Discussion

Prevention of this disease by control of intermediate or paratenic host is impractical and difficult to achieve, since the spectrum of intermediate and paratenic hosts is so diverse (Bailey 1972; Bowman 2009; Taylor et al. 2007), a situation similar to other severe parasitic diseases such as angiostrongylosis, which depends on a variety of molluscan intermediate hosts and vertebrate paratenic hosts (Bolt et al 1993, Ferdushy and Hasan 2010). Therefore control of *Spirocerca lupi* in the final host by medical prevention or therapy seems to be the only viable option.

Most studies in which macrocyclic lactones were tested for efficacy against *S. lupi* focused on therapeutic efficacy (Berry 2000; Lavy et al. 2002; Kelly et al. 2008), and although some treatment regimens have indicated promising preventative efficacy (Lavy et al. 2003; Kok et al. 2010; Le Sueur et al. 2010), this issue remains largely unresolved. The present study was designed to evaluate both preventative and therapeutic efficacy in experimentally infected animals. Assessment included not only oesophagoscopy but also faecal egg counts and quantification of pathological changes and worm numbers during necropsy.

In spite of the fact that dogs invariably vomited shortly after infection with the *S. lupi* L3 larvae, the establishment of the parasites was not negatively affected. The dogs in the negative control group were adequately infected with *S. lupi*, as concluded from the extensive damage caused by the worms in the wall of the thoracic aorta, the nodules formed in the oesophagus and the large numbers of worms recovered from the oesophageal nodules. The percentage establishment was closely similar to that reported by Bailey (1972), namely 45 % asopposed to 47 %, and was slightly higher than that reported by Kok et al. (2011). The adequacy of infection in the control group enabled a valid comparison of treated groups to the control group.

The use of oesophagoscopy to detect oesophageal nodules associated with *S. lupi* infections is a standard diagnostic procedure (Mazaki-Tovi et al. 2002) and has been used often in studies to determine the efficacy of therapeutic procedures in the treatment of progressed spirocercosis (Lavy et al. 2003; Kelly et al. 2008; Le Sueur et al. 2010; Lobetti 2000).

In the present study, endoscopy proved to be a valuable tool to determine whether or not oesophageal spirocercosis had already developed in dogs six months after the experimental introduction of L_3 larvae. The procedure confirmed that all dogs in the untreated control group and all but one of the dogs in group 3, until then untreated, had oesophageal nodules. This confirmation allowed investigators to go ahead with the therapeutic treatments of the animals in group 3. At the same time, the absence of any oesophageal lesions in the dogs in group 2 already indicated the preventative efficacy of the monthly treatments that had started before the first experimental infections.

In the follow-up endoscopic examinations of animals in group 3, nodules were still recorded as present in seven of the eight dogs 2–3 weeks before necropsy. However, at necropsy nodules were found in only three of the dogs. The authors suggest that because of the elasticity of the oesophagus, its mucosa is strongly folded; these folds tend to form small ridges which could have been mistaken for nodules that were not yet completely resolved. Researchers tended to record any ridges/swellings as nodules rather than missing any actual nodules, and were therefore likely to overestimate the number of nodules during such endoscopic examinations. On re-examination of video clips taken in this study during examinations, such ridges could mostly be identified as ridges and not as nodules.

Faecal egg counts proved to be supportive of the early endoscopic confirmation of patent infections in dogs belonging to groups 1 and 3, and the apparent absence of infections in dogs of group 2. Therapeutic efficacy of the treatment of dogs in group 3 was also indicated early after the start of treatment by the egg numbers that never increased to meaningful numbers (compared to those of the

control group) and gradually disappeared from the faeces. Lavy et al. (2002, 2003) reported on the value of egg counts in the assessment of therapeutic efficacy but le Sueur et al. (2010) did not obtain any useful results from faecal examinations.

As a first objective of the present study, one group of dogs was treated 30 days before the first experimental infection and monthly thereafter to determine whether or not the treatment would effectively prevent the establishment of *S. lupi* larvae in the thoracic aorta as well as the associated damage to the wall of the aorta. It was shown that for group 2 the efficacy of prevention was 100 %, based on the results of endoscopic visualisation, faecal examinations and most importantly, necropsy examination of the thoracic aorta. The latter is the only assessment that can unequivocally determine whether or not the aorta was damaged. Kok et al. (2010) have shown that even in animals that responded positively to treatment for the prevention of oesophageal spirocercosis, damage to the thoracic aorta still occurred and could only be quantified during post-mortem examination of the aorta. Kirberger et al. (2013) have shown that computed tomographic and radiographic procedures can be used to detect aortic lesions but these are likely to be of value mainly when gross lesions are present.

These results obtained during an experimental infection confirm those of Le Sueur et al. (2010) who reported on the high efficacy of an imidacloprid 10 %/moxidectin 2.5 % spot-on to prevent infection with *S. lupi* in a large population of young dogs naturally exposed to *S. lupi* on Réunion Island.

As a second objective of the study the dogs in group 3 were treated for the first time at about 6 months post infection, when it had been confirmed by endoscopic examination that seven out of the eight dogs already had oesophageal spirocercosis. The dogs were then treated weekly thereafter to determine whether or not the treatment would effectively reduce the number of *S. lupi* adults inhabiting the oesophageal nodules and at the same time resolve the nodules. Nineteen weekly treatments were given over a period of 128 days and necropsy followed 14 days later. Only small nodules remained in the oesophagus of three of the dogs and only three *S. lupi* worms were recovered from two of the dogs (efficacy of treatment calculated as 98.5 % against the adult worms). As was expected, damage to the aorta of dogs in group 3 did not differ from that seen in the untreated control dogs. Even when dogs recover from oesophageal spirocercosis, they remain at risk because of the irreversible damage to the aorta. Sudden death may thus still occur in case of a ruptured aorta. This underlines the importance of prevention of infection, in order to avoid extensive damage of the aorta caused during the development of the parasite.

A number of studies have been conducted with the aim to determine therapeutic efficacy of macrocyclic lactones, notably doramectin and milbemycin oxime. In all of these studies, with either naturally or experimentally infected clinical cases, assessment was done with endoscopic examination of nodules to determine successful resolution. Resolution of nodules, not necessarily complete, occurred after 42–84 days (Berry 2000), 35–544 days (Lavy et al. 2002), 60–180 days (Mylonakis et al. 2004) and 95–186 days (Kelly et al. 2008). Doramectin was mostly injected subcutaneously at 200 to 400 µg/kg at 14-day to monthly intervals for up to 20 treatments. However, Mylonakis et al. (2004) also gave daily oral doses of doramectin to two dogs, and Lobetti (2012) followed this procedure in a study with 20 naturally infected dogs dosed daily with 500 µg/kg oral doramectin and reported the resolution of oesophageal nodules after 42–126 days. Kelly et al. (2008) dosed dogs with oral milbemycin oxime.

In the present study, resolution of oesophagel nodules was almost complete after 19 weekly spot-on treatments with imidacloprid 10 %/moxidectin 2.5 % (Advocate®, Advantage Multi®, Bayer) over a period of 128 days. Only three live worms were recovered from the treated group as opposed to 144 from the control group (efficacy 98.5 %).

The product used in this study contains a combination of imidacloprid and moxidectin in concentrations of 10 % and 2.5 %, respectively. Imidacloprid

is a topically applied, non-systemic insecticide belonging to the chloronicotinyl class of compounds. It was first discovered in 1984 and introduced onto the market in 1991, its main function in veterinary medicine being the control of fleas on dogs and cats (Mencke and Jeschke 2002).

Moxidectin is a pentacyclic lactone endectocide of the milbemycin class of compounds, chemically derived from the macrocylic lactone nemadectin, first isolated in 1983 as a fermentation product of the bacterium *Streptomyces cyaneogriseus* (Prichard et al. 2012; Rock et al. 2003). Moxidectin has an extremely high lipophilicity, approximately 100 times higher than that of ivermectin, and this contributes to its very high volume of distribution (Hennessy and Alvinerie 2003). These factors, combined with an elimination half-life of 19 days in the dog (Vanapalli et al. 2002), give moxidectin an extended post-administration residual action in the body.

Macrocyclic lactones exert their anthelmintic effect by binding to glutamate-gated chloride channels, which are expressed on nematode neurones and pharyngeal muscle cells. The net effect of this binding is an irreversible opening of these channels, leading to prolonged hyper- or depolarisation of the nerve cell, preventing further functioning and ultimately leading to paralysis (Wolstenholme and Rogers 2005).

When looking at the life cycle of *S. lupi*, it stands to reason that the ideal product for preventing and treating infections with this parasite should present a high level of efficacy against immature and mature stages of this nematode, a high volume of distribution and an extended period of action in order to be effective. Other important product attributes would include safety in target species as well as ease of administration.

It is evident that different macrocyclic lactones are promising in the prevention and therapeutic treatment of spirocercosis. Disadvantages are that products are often not licensed and may require high doses or administration by a veterinarian. In this regard, Kelly et al. (2008) emphasised the practical advantages of oral doses of milbemycin oxime. The convenience of using the tested formulation as a spot-on formulation in either weekly or monthly treatments exceeds that of any other product hitherto used. Furthermore, the efficacy of imidacloprid 10%/moxidectin 2.5% against migrating and encapsulated phases of *S. lupi* was proven conclusively in the present study.

At no time during the study any adverse reaction to the topically applied product was noted. A total of 19 treatments were applied weekly to the treatment group 3 with no manifestation of any undesirable side effects. It must be stated that none of the dogs used in this study belonged to the collie or any related breeds which are known for their predisposition to defects of the MDR I gene and subsequent susceptibility to neurological macrocyclic lactone toxicity (Mealey 2004; Mealey et al. 2001).

In July 2012, imidacloprid 10%/moxidectin 2.5% (Advocate®, Bayer) was officially licensed in South Africa for the prevention and treatment of *Spirocerca lupi* infections based on the results of this study, and to the authors' best knowledge became the first product in the world to receive this indication.

Ethical standards

All institutional and national guidelines for the care and use of laboratory and study animals were followed.

Conflict of interest

This clinical study was funded by Bayer South Africa (Pty) Ltd and Bayer Animal Health GmbH, Leverkusen, Germany, of which Clinton Austin and Roland Schaper are employees. ClinVet is an independent, South African Contract Research Organisation contracted to manage the conduct of the study. Dawie J Kok and D Crafford are employees of Clinvet.

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